In the Specification

On page 72, lines 15 and 17, delete "isobestic" and substitute therefor ---isosbestic---.

In the Claims

Cancel Claims 42, 45, 64, 67 and 68. Amend Claims 63, 65 and 66 as shown below.

63. (Amended) A method for producing [SNO-hemoglobin] SNO-oxyhemoglobin, said method comprising mixing nitric oxide and deoxyhemoglobin at pH 7.4 and exposing the resulting solution to air.

65. (Amended) A method for producing S-nitrosohemoglobin, said method comprising mixing nitric oxide and oxyhemoglobin at a heme:NO ratio of less than about 10 in aqueous buffer at pH 7.4.

66. (Amended) The method of Claim 65 wherein the [oxyhemoglobin is greater than about 18 μM] heme:NO ratio is greater than about 100.

REMARKS

Claims 42, 45, 64, 67 and 68 have been canceled. Claims 63, 65 and 66 have been amended.

Rejection of Claims 63-66 Under 35 U.S.C. § 112, First Paragraph (Item 2, Page 3 of Office Action)

Claims 63-66 have been rejected under 35 U.S.C. § 112, first paragraph, "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s), at the time the application was filed, had possession of the claimed invention."

Applicants assume that the initial sentence of the rejection was meant to read, "Claims 63-*68* are rejected under 35 U.S.C. 112, first paragraph . . . ." (Emphasis added.) Claims 67 and 68, as well as Claims 63-66, are discussed in the rejection.

It is said that "[t]he specification disclosure (bottom of page 72 - top of 73) of a specific multistep protocol for forming SNO-hemoglobin does not support the generic method claim 63."

Applicants do not see what essential features of the method for producing SNO-hemoglobin are missing from the claim. The method entails starting with a solution of deoxygenated hemoglobin. Hemoglobin can be conveniently stored in a buffer compatible with physiological activity. One of ordinary skill in the art would be able to choose any number of suitable buffers appropriate for the stability and physiological activity of hemoglobin. Deoxygenation of a solution of hemoglobin is something which one of ordinary skill in the art needs no further instruction to carry out. Also required is nitric oxide, a gas at room temperature and at body temperature, so that it is ordinarily dissolved in an aqueous solution for use in experiments on its biological effects. The claim requires that the deoxygenated hemoglobin and the nitric oxide be brought in contact with each other, and that the resulting solution be exposed to air, the simplest method to oxygenate the hemoglobin.

It is said by the Examiner that, "[t]he specification disclosure (bottom of page 73 - top of 74) and especially page 73, lines 14-20 which disclose a specific multistep protocol for making nitrosylhemoglobin, does not support the generic method of claim 64, nor does the specification support the 'heme:NO ratio of greater than about 14' as presently claimed."

Claim 64 has been canceled.

The Examiner states, "The specification disclosure (bottom of page 75 - top of page 76) which discloses a specific multistep protocol for making SNO-Hb which includes specific buffer and amounts does not support the generic method of new claim 65." The "specific multistep protocol" consists of one step as given in Claim 65. Claim 65 has been amended.

The Examiner states that "specific support for 18.3 μM hemoglobin fails to support the range of "greater than about 18 μM" in dependent claim 66." Claim 66 has been amended to specify a heme:NO ratio.

The Examiner states, regarding Claim 67, that [t]he specification disclosure in Example 16 (page 73) discloses a specific multistep method of making predominantly nitrosylhemoglobin which requires specific: hemoglobin A amounts, buffer and amounts thereof; pH (e.g. 7.4); timing etc." Claim 67 has been canceled.

The Examiner states, "Additionally, the claimed heme-ratio of 'at least about 70:1' in both claims 67 and 68 is not supported by the disclosed 'approximately 7:1' since these terms appear to possess different scope e.g. the claimed invention is broader."

Claims 67 and 68 have been canceled.

The Examiner states that the composition of Claim 68 is not supported by the specification. Claim 68 has been canceled.

Rejection of Claims 10-15, 42, 45, 46 and 63-68 Under 35 U.S.C. § 112, First Paragraph (Item 3, Page 4 of Office Action)

Claims 10-15, 42, 45, 46 and 63-68 have been rejected under 35 U.S.C. § 112, first paragraph, as they are said to be "based on a disclosure which is not enabling." It should be pointed out that the specification includes description of an assay that can be used to detect SNO-hemoglobin, whether it is in the form of oxy-, deoxy- or met-hemoglobin. See methods sections, page 66, line 11 to page 68, line 4. Thus, one of ordinary skill in the art can determine, using the examples in the specification as starting points, operable variations in the procedures (e.g., in pH, in buffer, in reagent and concentrations thereof) to produce SNO-hemoglobins. As explained also in the specification, spectrophotometry can be used to detect changes in the hemoglobin at the heme.

Claims 42, 45, 64, 67 and 68 have been canceled.

Methods for producing SNO-oxyhemoglobin (pertaining to Claims 10-12, 63, 65 and 66) are described, for instance, on page 47, line 14 to page 48, line 13. Also see Example 11, page 69, line 21 to page 71, line 5. This description includes the results that can be expected to occur at various ratios of reagent to hemoglobin.

Methods for producing SNO-deoxyhemoglobin (pertaining to Claims 13-15) are described, for instance, on page 47, lines 1-13 and page 47, line 31 to page 48, line 24. This description includes the results that can be expected to occur at various ratios of reagent to hemoglobin.

The quotation from page 77, lines 25-30 of the specification refers to methods of producing SNO-oxyhemoglobin from incubations of oxyhemoglobin with nitric oxide (relevant to Claims 46, 65 and 66. Example 21 (page 76, line 26 to page 77, line 39) gives guidelines on buffers that were used successfully and others to be avoided to minimize oxidation. Example 20

(page 76, lines 7-25) gives additional information on the effect of protein concentration on the yield of oxidized hemoglobin, if it is desired to minimize formation of oxidized hemoglobin.

One of ordinary skill in the art would know that deoxyhemoglobin can only be maintained in the absence of oxygen, and that hemoglobin, if it is not oxidized (methemoglobin), will be oxyhemoglobin if exposed to air. It is not necessary to explicitly state this in the claims.

Rejection of Claims 45 and 68 Under 35 U.S.C. § 112, Second Paragraph (Item 5, Page 5 of Office Action)

Claims 45 and 68 have been rejected under 35 U.S.C. § 112, second paragraph , as they are said to be "indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention."

Claims 45 and 68 have been canceled.

Rejection of Claims 10, 11, 13, 14, 43, 44, 63, 65 and 66 Under 35 U.S.C. § 102(b), Or, Alternatively, Under 35 U.S.C. § 103(b) (Item 6, Page 6 of Office Action)

Claims 10, 11, 13, 14, 43, 44, 63, 65 and 66 have been rejected under 35 U.S.C. § 102(b) as "being anticipated by or alternatively under 35 U.S.C. § 103 as being obvious over Chem. Res. Tox. 1990 Vol. 3, pages 289-291."

Wade, R.S. and C.E. Castro (*Chem. Res. Tox. 3*:289-291, 1990; reference AT5) describe a reaction of nitrosoamines with heme to produce nitrosyl-heme products (Equation 1), a reaction of hemoglobin or myoglobin with nitric oxide to produce FeII-NO adducts (first paragraph), a reaction of metmyoglobin with nitric oxide to produce the heme-NO adduct (Equation 2), and the corresponding heme-NO adducts of the other heme proteins listed in Table 1 on page 289. Wade and Castro also describe (Equation 3) a reaction in which oxidized heme proteins, which are unable to carry oxygen, react with NO and a molecule providing a nucleophilic site (e.g., phenol), to produce a nucleophile-NO product (e.g., nitrosophenol), and the nitrosyl-heme protein. Nowhere does the reference describe a stable product of a reaction between a species of nitric oxide with a site on hemoglobin other than the heme Fe. The cited claims pertain to SNO-hemoglobin, an S-nitrosyl product, not an Fe-nitrosyl product. There is no suggestion in the reference that SNO-hemoglobin is possible to produce, and no suggestion of how to produce any product other than a heme-NO product in which NO is bound to the heme Fe. Therefore, Claims

10, 11, 13, 14, 43, 63, 65 and 66 are not anticipated by, and not rendered obvious by, the cited reference.

Rejection of Claims 42 and 45 Under 35 U.S.C. § 102(b) (Item 7, Page 7 of Office Action)

Claims 42 and 45 have been rejected under 35 U.S.C. § 102(b) "as being anticipated by Moore et al., J Biol. Chem. Vol. 251, No. 9, (5/76) pages 2788-2794."

Claims 42 and 45 have been canceled.

Rejection of Claims 42 and 45 Under 35 U.S.C. § 102(b) (Item 8, Page 7 of Office Action)

Claims 42 and 45 have been rejected under 35 U.S.C. § 102(b) as they are said to be "anticipated by Sharma et al., J. Biol. Chem. Vol. 253, No. 18 (9/78) pages 6467-72."

Claims 42 and 45 have been canceled.

Rejection of Claims 16, 20-22, 27-28 and 40 Under 35 U.S.C. §103(a) (Item 9, Page 8 of Office Action)

Claims 16, 20-22, 27-28 and 40 have been rejected under 35 U.S.C. §103(a), as the claims are said to be "obvious over Stamler et al., WO 93/09806 (5/93)."

The Examiner states, "Similarly to low molecular weight thiols, the Stamler reference further teaches that proteins (including hemoglobin), which are nitrosylated on oxygen, carbon or nitrogen sites possess the same therapeutic utility as nitrosylated/nitrated low molecular weight thiol compounds (e.g. see page 6, lines 13-15; page 7, lines 17-21; and claims)." The Examiner further states:

> Additionally, the use of nitrosated/nitrated proteins, including nitrosated/nitrated hemoglobin to deliver NO to tissues (e.g. claim 40) in order to effectuate the treatment of abnormalities or diseases which are mediated by nitric oxide and oxygen metabolism (e.g. lung disease, sickle cell anemia, heart disease, high blood pressure etc.) would have been obvious since the reference discloses the use of nitrosated proteins, including nitrosated hemoglobin, to treat such disease states.

From these statements, it appears that the Examiner draws the conclusion from WO 93/09806 that a number of nitrosylated species of hemoglobin were prepared, and that these species had been used, or were proposed to be used, in methods of therapy for diseases mediated by nitric oxide metabolism.

The existence of all but one species of nitrosylated hemoglobin -- nitrosylhemoglobin, (which has the NO on the heme iron and cannot carry and donate NO) -- was unknown before the invention. No person of ordinary skill in the art would be able to conclude from WO 93/09806 that any other species of nitrosated or nitrated hemoglobin had been synthesized. Example 19 of WO 93/09806 presents a number of errors in reporting the procedures that were followed, in the logic applied to the results, and in the conclusions that were drawn from those results. These errors are described in the paragraphs that follow.

One matter is the missing reagent on line 5 of page 58 of WO 93/09806. Although the context of Example 19 might suggest that the reagent is SNOAc (*S*-nitroso-*N*-acetylcysteine), the laboratory notebook records of Dr. Stamler show that the reagent used for the experiment, the results of which are shown in Figure 28, was actually acidified nitrite. See Exhibits A and B with the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 filed with the United States Patent and Trademark Office on January 6, 1999.

If we assume that the reagent was SNOAc, then the Saville assay as described in Example 19 of WO 93/09806 could not have yielded interpretable results. Example 19 includes no report of a separation step to separate the reagent, a low molecular weight S-nitrosothiol, from the product, which the Examiner presumes to be SNO-hemoglobin, a high molecular weight protein. Thus, the assay would give the misleading result, with SNOAc, and in all such cases where a low molecular weight S-nitrosothiol is used as a reagent, that an S-nitrosothiol product was formed, as the assay would detect the reagent. In fact, no separation step was performed, as Dr. Stamler has stated in the Declaration. See the second paragraph of statement 5 of the Declaration.

A minor matter is the number given as the absorption maximum of 450 nm as reported on line 14, for Figure 28. It can be seen from Figure 28 that this maximum is 540; one might assume the units are nanometers for the x-axis; no units are given for the y-axis. However, a more important matter is that it is not reported what solution produced this spectrum. It is not recognizable as a spectrum of hemoglobin. SNO-hemoglobin has no characteristic spectrum that is distinguishable from any other species of hemoglobin, as one of skill in the art would know. If the spectrum in Figure 28 is intended to be that of the azo dye which is generated from nitrosothiol in the Saville assay, it is not informative, as the Saville assay requires measurements made both in the presence and absence of mercuric ions, to assay for S-nitrosothiol.

The sentence at lines 15-16 of page 58 of WO 93/09806 makes no sense, as the "S-nitrosothiol bond formation" being referred to in this sentence is supposed to be in SNO-hemoglobin, and could not be "demonstrated" in any way by "using NO^+ equivalents in the form of SNOAC."

The conclusions of the second paragraph on page 58 of WO 93/09806 are wrong. It is impossible to tell from Figure 29 which line of the spectrum in the region of approximately 540-580 nm can be attributed to a particular synthesis procedure, as the lines of the five different spectra in the figure are not identified, and the lines of the spectra overlap. In any case, that region of the hemoglobin spectrum is difficult to interpret in general, as several species of hemoglobin absorb in that range. Note, for example, that the spectrum of NO(FeII)hemoglobin in the region of 540-580 nm resembles that of oxyhemoglobin. See Exhibit Z. Thus, the relative contributions of each of these species to the various spectra cannot be determined from the figure. What is clear, however, is that Figure 29 shows multiple hemoglobin derivatives in which the redox metal sites are different.

Figure 30, referred to on page 58, lines 19-25 of WO 93/09806, is said to be the spectrum of nitrosyl-hemoglobin. However, Figure 30 is not helpful for purposes of comparison with any of the other spectra, being plotted as a separate graph.

Lines 23-25 of page 58 draw the conclusion, "The fact that the S-nitrosothiol did not react with the redox metal site of hemoglobin, but with its thiol group instead, indicates that the reactive NO species donated by the S-nitrosothiol is nitrosonium or nitroxyl." No such conclusion can be drawn. On the contrary, one skilled in the art would conclude that there *was* a reaction with the redox metal site of hemoglobin. There is no evidence, from any of the assays or spectra examined, that S-nitrosothiol groups are present on a hemoglobin product, and no conclusion can be made about any reactive NO species from any of the experiments that might be described in Example 19.

Lines 26-27 of page 58 state, without any evidence, "S-nitrosylation of hemoglobin does not result in the formation of methemoglobin and consequent impairment in hemoglobin-oxygen binding." On the contrary, methemoglobin is definitely formed by the processes one might assume to be described in Example 19, as explained below.

The sentence on page 58, line 28 to page 59, line 1 describes "a leftward shift in the hemoglobin-oxygen association curve." This leftward shift was merely a result of the presence

of methemoglobin. Experiments to produce a hemoglobin-oxygen association curve are done in a tonometer of various oxygen partial pressures. The presence of substantial amounts of methemoglobin (λ_{max} = 405 nm) are well known to cause this shift. This has been misinterpreted by the drafters of WO 93/09806 as an increase in oxygen binding. The first sentence on page 59 continues, "Thus, the reaction between S-nitrosothiols and hemoglobin not only eliminates the inhibition of oxygen binding which occurs from the reaction with uncharged NO and generation of methemoglobin, but it actually increases oxygen binding." In the absence of any evidence of a reaction between S-nitrosothiols and the thiols of hemoglobin, the skilled person could not conclude what effect SNO-hemoglobin might have on oxygen binding.

The interpretation of hemoglobin spectra is not straightforward because of the many hemoglobin species with similar spectra. See, for example, the spectra of NO-hemoglobin in the Soret region and in the visible range (Exhibits Y and Z, respectively, which were produced in the laboratory of Dr. Stamler). There are, therefore, several alternative explanations that are more plausible to one of skill in the art, but all of them include reactions taking place at the metal redox center. None of the spectra of Figure 29 can be identified as being attributable to any one species of hemoglobin. To get an accurate measurement of the relative contributions of oxy-, NO(FeII)- or met-hemoglobin species among a mixture of hemoglobin species, it would be necessary to determine the amounts of the species oxy-, NO(FeII)-, and met-hemoglobin, using methods such as those reported by Gow, A.J. *et al., Proc. Natl. Acad. Sci. USA 96*:9027-9032, 1999 (Exhibit X). This was not done in this case, and these methods were not known at the time of the publication of WO 93/09806. However, it is clear that the products from all of the procedures 1-4 (as these procedures are summarized on Exhibit C with the Declaration of Dr. Stamler) include significant amounts of one or more species of hemoglobin that cause a leftward shift in the peaks of the Soret region of the spectrum. It is difficult to determine the exact peaks, because the measurements were not made, or not given in Example 19. However, it is clear that the leftward shift from the "middle spectra" to those in spectra 3 and 4 is at least 5 nm, and that, overall, the peaks in the Soret region cover a range of at least 10 nm. The explanation for the leftwards shift is the presence of significant methemoglobin, as the maximum absorbance for oxyhemoglobin is about 415 nm, the maximum for NO-hemoglobin is about 417 nm, and the maximum for methemoglobin is about 405 nm. (The maximum for deoxyhemoglobin is 430 nm. At least one of the spectra would appear to have a partial deoxy component that would greatly

confound the interpretation.) For procedures 1 and 2, the product has a maximum in the Soret region at 417 nm, which may indicate the presence of NO(FeII)hemoglobin, and for procedures 3 and 4, in which a higher concentration of SNOAc was used, there has been a leftwards shift, indicating that a major product is methemoglobin. The spectra cannot tell one of skill in the art anything about whether a reaction occurred at the thiols of the cysteine residues of hemoglobin; only that reactions are definitely occurring at the redox metal center.

An alternative explanation for the leftward shift in the hemoglobin absorbance spectrum is that the methemoglobin present promotes the R (high affinity) structure. That is, if the hemes of one or more subunits of the hemoglobin tetramer are oxidized, the remaining subunits bind oxygen more readily than they would if the subunits were in the deoxy (T structure) state.

In any case, the experiment of procedures 1 and 2 of Exhibit C has been repeated, and the result was that, by an improved Saville assay modified from that known at the time of the publication of WO 93/09806, no SNO-hemoglobin was produced. See statement 5 of the Declaration of Dr. Stamler, and accompanying Exhibits E1-E3. Figure 29 is, therefore, uninterpretable, but the conclusion of one of ordinary skill in the art could not be, seeing the maximum absorbance leftward shifted as it is, that (page 58, lines 17-19) "the UV spectrum of hemoglobin incubated with SNOAC shows no reaction at the redox metal (iron-binding site) of hemoglobin, over 15 minutes."

As can be seen from consideration of the above several points, the disclosure of WO 93/09806 contains statements that are in error, as well as statements and figures that are inconsistent and uninterpretable. Therefore, it cannot be said that WO 93/09806 discloses SNO-hemoglobin or any other form of nitrosated or nitrated hemoglobin to the person of ordinary skill in the art.

Stamler *et al.* (WO 93/09806) disclose S-nitroso-proteins, in particular, S-nitroso-tPA (tPA is tissue plasminogen activator), S-nitroso-BSA, S-nitroso-cathepsin B, S-nitroso-lipoprotein and S-nitroso-immunoglobulin, and methods for producing the same, using NO or $NaNO_2$ as the reagent under acidic conditions. They also report a method which they claim results in the synthesis of S-nitroso-hemoglobin. However, this compound was not produced by any method reported in WO 93/09806, as attested to in the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 submitted on January 6, 1999. Methods used to synthesize other S-nitroso-proteins, which might have been expected to nitrosate or polynitrosate hemoglobin,

dissociated hemoglobin into its subunits, oxidized the heme Fe and rendered the product pieces useless for carrying oxygen. Methods described in the specification that result in the synthesis of nitrosated hemoglobins are substantially different from the unsuccessful acidified nitrite method described in WO 93/09806.

Before the invention, it was known that low molecular weight nitrosothiols were desirable as vasodilators and platelet inhibitors. Low molecular weight thiols were not reported to have any biological activity on their own. Hemoglobins, with the heme Fe in an unoxidized state, were known to be carriers of oxygen. Before the invention, it was not known that any form of nitrosylated hemoglobin could function as a carrier and donor of NO (SNO-hemoglobin was totally unknown; nitrosylhemoglobin was known to bind NO extremely tightly). It was not known that NO could be transferred from SNO-hemoglobin to thiol, and from nitrosothiol to hemoglobin, without the transfer of NO adversely affecting the oxygen carrying function of hemoglobin. Therefore, before the invention, there was no motivation to combine a low molecular weight thiol or nitrosothiol with hemoglobin or nitrosated hemoglobin, as in Claim 16.

The reference suggests that S-nitrosoproteins be used for the delivery of NO to the tissues. However, SNO-hemoglobin was unknown before the time of Applicants' first synthesis of SNO-hemoglobin, and the properties observed for SNO-hemoglobin were different from those observed with other nitroso-proteins. (Whereas other SNO-proteins dilate blood vessels, SNO-oxyhemoglobin constricts blood vessels. See the written description at page 57, lines 3-14, and Figure 4A). Therefore, the use of SNO-hemoglobin in methods of therapy such as those defined in Claims 20-22, 27, 28 and 40 could not have been suggested by the reference.

Before Applicants' invention, nitrosylhemoglobin was known, but it was not known to be a "nitrosyl-containing donor of NO." Nitrosylhemoglobin was known only as a stable form of hemoglobin that was not useful for the carrying and delivery of oxygen, or for the delivery of NO, making it undesirable in methods of therapy. The prior art had discussed nitrosylhemoglobin as being physiologically important as a molecule that bound to NO, keeping it out of circulation (Wennmalm Å., *et al., Br. J. Pharmacol. 106*:507-508, 1992; reference AU4). Applicants, as is described for instance in Example 15, page 72, first discovered that nitrosylhemoglobin can be converted to SNO-hemoglobin under physiological conditions, and that nitrosylhemoglobin is, therefore, useful as a donor of NO. Before the discoveries of Applicants, it could not have been predicted that any modified form of hemoglobin might have

the effects of vasodilation and inhibition of platelet deposition, as unnitrosylated hemoglobin was known to have exactly the opposite effects (see, for example, the abstract and first paragraph in Olsen, S.B. et al., *Circulation 93*:327-332, 1996; copy provided as Exhibit W).

Rejection of Claim 41 Under 35 U.S.C. § 103(a) (Item 10, Page 9 of Office Action)

Claim 41 has been rejected under 35 U.S.C. § 103(a), as it is said that it is "unpatentable over Stamler WO 93/09806 as applied to claims 16, 20-22, 27-28 and 40 above, and further in view of Moore et al., J. Biol. Chem. Vol. 251, No. 9, (5/76) pages 2788-2794 or Sharma et al., J. Biol. Chem. Vol. 253, No. 18 (9/78) pages 6467-72."

The teachings of WO 93/09806 have been described above.

The Moore *et al.* and Sharma *et al.* (*J. Biol Chem. 253*:6467-6472, 1978) papers both describe studies on nitrosylhemoglobin and nitrosylmyoglobin (both produced from the respective deoxy molecules), in which dissociation of NO from these molecules is followed spectrophotometrically in the absence of oxygen. No studies of nitrosylhemoglobin and nitrosylmyoglobin are done under physiological conditions. The Moore *et al.* and Sharma *et al.* papers do not report or suggest any physiological effect of nitrosylhemoglobin or any other nitrosyl-heme containing NO donor. What was known about nitrosylhemoglobin from the prior art was that it is a *scavenger* of NO. See, for example, Greenburg, A.G. and H.W. Kim, *Art. Cells, Blood Subs., and Immob. Biotech. 23*:271-276, 1995, especially fifth paragraph on page 272; reference AX.

The Examiner bases the rejection primarily on the Stamler reference WO 93/09806, which he states "as disclosing the use of nitrosyl-heme containing NO donors to deliver NO or its biological equivalent to tissues...." The Examiner does not point out where this conclusion comes from. There is no such teaching in WO 93/09806 regarding nitrosyl-heme containing NO donors, and nitrosylhemoglobin in particular, or of any possible function nitrosyl-heme containing donors might have to deliver NO or its biological equivalent.

Combining the references, one of ordinary skill in the art seeking a method to deliver NO or its biological equivalent to tissues in an animal or human, might turn to an S-nitrosoprotein or other S-nitrosothiol as described in WO 93/09806 to be administered to a human or animal. However, one of ordinary skill in the art would know from other references in the literature that NO is extremely tightly bound to the heme Fe in nitrosylhemoglobin, and that

nitrosylhemoglobin was not a carrier of oxygen, and was not known to be a donor of NO or an intermediate in the formation of SNO-hemoglobin.

Rejection of Claims 10-15 Under 35 U.S.C. § 103(a) (Item 11, Page 10 of Office Action)

Claims 10-15 have been rejected under 35 U.S.C. § 103(a), as they are said to be "unpatentable over Stamler (WO 93/09806)."

The Examiner further states that "Stamler discloses different methods for thiol nitrosylation of proteins (as disclosed on page 30-31) which include:
1. reaction of nitrosylating agent (e.g. equimolar amounts of acidic $NaNO_2$ as nitrosating agent in a buffered saline at pH 7.4 for tPA):
2. exposure of the protein (e.g. tPA to NO gas in buffered saline)."
The Examiner also states that the "reference methods for thiol nitrosylation fail to disclose the use of 'excess' nitrosating agent, and preferably the selection of a low molecular weight S-nitrosothiol as the nitrosating agent for thionitrosylation of hemoglobin."

It should be pointed out that the acidified nitrite procedure of nitrosylation of proteins was not carried out at pH 7.4, as the Examiner concludes, but at a far more acidic pH, in 0.5 N HCl. Then, after the $NaNO_2$ had reacted and was no longer available, the resulting solution was brought to pH 7.4 by addition of NaOH and Tris buffered saline. Importantly, a nitrosylating reaction using $NaNO_2$ would not be successful at pH 7.4; $NaNO_2$ will only work under acid pH conditions. At either pH, the product of incubating $NaNO_2$ with hemoglobin would be oxidized.

The second method the Examiner states as disclosed in WO 93/09806 (exposure of the hemoglobin protein to NO gas in buffered saline, especially *excess* nitrosating agent) does not produce SNO-hemoglobin. That it is necessary to use a *low* ratio of NO:heme is demonstrated in the continuation-in-part application 08/874,992.

The teachings of WO 93/09806 have been discussed above. WO 93/09806 discloses methods of thiol nitrosylation that were apparently successful for the production of some SNO-proteins. However, as presented by Applicants previously, nowhere is it disclosed in the prior art, including WO 93/09806, that these methods were successful in any synthesis of SNO-hemoglobin at any pH. Hemoglobin is susceptible to dissociation of its subunits, denaturation, and oxidation of the heme Fe, and one of ordinary skill in the art would expect (as Applicants have confirmed experimentally as sworn to in the Declaration of Jonathan S. Stamler, M.D.

Under 37 C.F.R. § 1.132 mailed to the Patent Office on January 6, 1999, and as stated to the Examiner personally in the interview of August 5, 1999) that hemoglobin would suffer these effects from treatment with acidified nitrite in 0.5 N HCl.

One of ordinary skill in the art would know from other references in the prior art that, whereas tPA does not have heme groups, hemoglobin does, and that deoxyhemoglobin incubated with gaseous nitric oxide becomes nitrosylhemoglobin, in which NO is stably bound at the heme, and that oxyhemoglobin incubated with gaseous nitric oxide is oxidized to methemoglobin.

The Examiner states:

> But the Stamler reference (e.g. Example 19 on pages 58-59) specifically discloses the preferential selection of a low molecular weight S-nitrosothiol (e.g. SNOAc) instead of acidic $NaNO_2$ as utilized for tPA due to reduced ability of the SNOAc as compared with acidic nitrate to bind at the redox metal which reduces oxygen binding affinity.

As stated previously, WO 93/09806 does not show the successful modification of hemoglobin to form SNO-hemoglobin. No spectrophotometric evidence is presented that would indicate the presence of SNO-hemoglobin. No results are presented from what is referred to in Example 19 as "standard methods for detection of S-nitrosothiols (Saville, *Analyst 83*:670-672 (1958))." In fact, the Saville assay was not routinely used for the detection of S-nitrosothiols, but is a method for the quantitation of thiols that must be modified and adapted for effective use with SNO-hemoglobin. See the specification at page 67, line 11 to page 68, line 4. These modifications were not indicated in Example 19 of WO 93/09806 and would not be apparent to one of ordinary skill in the art. Thus, the Saville assay as referred to in WO 93/09806 would not have yielded any interpretable results. With no evidence that mixing SNOAc and hemoglobin produces any product, one of ordinary skill in the art would conclude that there is no reasonable expectation of success of any method to produce SNO-hemoglobin given in WO 93/09806.

The Examiner states, "Further, the use of 'excess nitrosating agent' in either reaction 1 or 2 above is suggested by the Stamler reference since providing a greater concentration of NO serves to enhance the therapeutic efficacy of the nitrosylated proteins (e.g. see bottom of page 23 to top of page 24)." WO 93/09806 does not suggest that the use of greater relative amounts of nitrosating agent or greater concentrations of NO. WO 93/09806 discusses at pages 23 and 24 the advantage of molecules modified at multiple sites with NO, but does not discuss or suggest any method of synthesis of such molecules, including the addition of excess nitrosating agent,

nor does it suggest any changes in reaction conditions to add NO groups at multiple sites on hemoglobin. Contrary to what the Examiner suggests, the use of higher ratios of nitrosating agent to hemoglobin does not necessarily "serve to enhance the therapeutic efficacy" of a nitrosylated hemoglobin or even lead to higher higher yields of SNO-hemoglobin product. For example, see the written description at page 75, line 29 to page 76, line 6, and Figure 17, wherein it is shown that higher NO:heme ratios decrease the efficiency of the reaction with oxyhemoglobin to produce SNO-hemoglobin. Also see Figures 18A, 18B and 19, which show that higher ratios of NO:hemoglobin result in increasing formation of methemoglobin.

The Examiner further states:

> It is further noted that the use of higher pH values (e.g. pH 7.4) than that utilized in the thionitrosylated hemoglobin example (e.g. pH 6.9 Example 19) is also suggested by the reference since thionitrosylated proteins are known to be stable under physiological conditions (e.g. TBS, pH 7.4, room temperature: see page 31) and further the reference discloses the use of pH 7.4 in the steps analogous to that of Example 19: see page 30, lines 20-27; page 33, lines 20-26).

WO 93/09806 does not suggest the use of any reaction that can be carried out at pH 7.4. The reactions described on page 30, lines 20-27 and page 33, lines 20-26 were carried out at a very low pH, in 0.5 N HCl. The reactions were later neutralized with NaOH and brought to pH 7.4. Attempts to nitrosylate a protein with $NaNO_2$ at pH 7.4 would be unsuccessful. The Examiner's statements regarding the stability of thionitrosylated proteins under physiological conditions (e.g., pH 7.4) are not understood, as S-nitrosothiols are known to be more stable in acid than at neutral or alkaline pHs.

It is not understood how one can "optimize" reaction conditions, as the Examiner suggests is within the skill of one of ordinary skill in the art, if no successful reaction conditions have been demonstrated at all.

Rejection of Claims 17-19 and 29 Under 35 U.S.C. § 103(a) (Item 12, Page 12 of Office Action)

Claims 17-19 and 29 have been rejected under 35 U.S.C. § 103(a) as being "unpatentable over Feola et al., U.S. Pat. No. 5,439,882 (8/95; filed 5/93 or earlier) and Stamler, and if necessary further in view of Moore or Sharma."

Applicants respectfully request clarification of the reasoning behind the combination of references cited in the rejection, as it is not understood what "if necessary" means. If there are

alternatives in the reasoning, Applicants request that they be written out, so that there is opportunity for Applicants to respond to every rejection.

Feola *et al.* (US 5,439,882) describe a cross-linked mammalian hemoglobin, a method of making the same, and a method of using the same as a blood substitute. Reduced glutathione, a thiol that occurs naturally in red blood cells, is only used in the method of synthesis to stop the cross-linking of hemoglobin when using o-adenosine as a cross-linking agent; in this case glutathione reacts through its amine to become cross-linked to a second glutathione molecule or to become cross-linked to hemoglobin. Excess glutathione is dialyzed out, so that the cross-linked hemoglobin composition contains no free low molecular weight thiol. Thus, the Examiner's conclusion that Feola's blood substitutes comprise "hemoglobin . . . combined with glutathione as a blood substitute" is incorrect. See column 13, lines 2-6 and lines 27-30, and column 18, lines 62-64. Feola *et al.* do not teach or suggest any form of nitrosated hemoglobin or suggest any advantage for it. Nor do Feola *et al.* teach or suggest any form of a nitrosyl-heme-containing donor of NO, such as nitrosyl-hemoglobin.

The teachings of Stamler (WO 93/09806), Moore *et al., J. Biol. Chem. 251(9)*:2788-2794 (1976) and Sharma *et al.*, (*J. Biol Chem. 253*:6467-6472, 1978) have been described above.

The Examiner states:

> The Stamler reference specifically discloses the use of nitrosylated proteins and low molecular weight nitrosating agents (e.g. see pages 1-2; page 24, lines 10-16) preparations thereof for the treatment of disorders by increasing oxygen capacity and transport; modulating CO and NO to tissues; scavenging radicals and vasodilation such as treating lung diseases (e.g. ARDS) and hypoxic disorders (e.g., see pages 19-25 and claims)."

Some of the properties attributed by the Examiner to nitrosylated proteins and low molecular weight nitrosating agents can only belong to nitrosylated forms of hemoglobin or perhaps some other heme proteins ("increasing oxygen capacity and transport; modulating CO and NO to tissues; scavenging radicals...."). As presented herein and in previous Amendments, SNO-hemoglobin is not disclosed in WO 93/09806, and other forms of nitrosylated hemoglobin and other heme proteins were not known to have any of these properties.

The Examiner states, "Thus, the Stamler *et al.* reference provides the skilled artisan with motivation to utilize nitrosated hemoglobin alone or with a low molecular weight S-nitrosothiol to make a blood substitute for treating sickle cell anemia in order to increase blood volume,

oxygen delivery and reduce vasoconstriction as effected by nitrosated hemoglobins alone or in conjunction with a nitrosothiol."

The claims cited in the rejection are drawn to a method for delivering NO in a mammal, comprising administering to the mammal an effective amount of a blood substitute comprising nitrosated hemoglobin, or comprising nitrosated hemoglobin and low molecular weight S-nitrosothiol, and to a method for scavenging oxygen free radicals and NO· in a mammal, comprising administering to the mammal an effective amount of a blood substitute comprising nitrosated hemoglobin, and to a blood substitute comprising nitrosated or nitrated hemoglobin. The Examiner's reasoning as to where motivation for the combination of references is to be drawn from is not understood, as the problem to be solved as presented in the claims is not that of treating sickle cell anemia. One of ordinary skill in the art wanting to provide a blood substitute useful in the treatment of sickle cell anemia might want to be able to deliver both oxygen and nitric oxide. Nitrosylhemoglobin would not have been desirable, as it is unable to deliver either oxygen or nitric oxide, and was thought to undergo oxidation to nitrate and methemoglobin. See, for example, Greenburg, A.G. and H.W. Kim, *Art. Cells, Blood Subs., and Immob. Biotech. 23*:271-276, 1995, especially fifth paragraph on page 272; reference AX. It could not have been known, from the prior art known before the invention, that one form of SNO-hemoglobin, SNO-oxyhemoglobin, which is capable of delivering oxygen, would have the unexpected effects of constricting blood vessels and activating platelets.

The Stamler *et al.* reference WO 93/09806 does not provide any reason or motivation why one might use hemoglobin or a nitrosated hemoglobin in combination with a thiol or nitrosothiol. There is no disclosure or suggestion of any added effect from the combination.

The instant application, however, does provide one of ordinary skill in the art with motivation to combine hemoglobin or nitrosated hemoglobin with thiol or nitrosothiol. See, for example, page 58, line 3 to page 59, line 15, wherein it is shown that NO is transferred from SNO-hemoglobin to glutathione. NO can, by this mechanism, be exported from the red blood cells to the tissues. One of ordinary skill in the art would be motivated, from the specification, to include a thiol or nitrosothiol with hemoglobin or nitrosated hemoglobin, for the potentiation effect as observed with SNO-hemoglobin and glutathione.

The Examiner further states, "Further, nitrosylated hemoglobin preparations, e.g., nitosylhemoglobin compositions, are conventionally known in the art (e.g., see the Moore and Sharma references)."

As discussed above, the Moore and Sharma references disclose studies on nitrosylhemoglobin (in which NO is bound to the heme Fe) and no other nitrosated forms of hemoglobin (those having NO bound at a thiol or any other sites), but do not disclose any physiological function for nitrosylhemoglobin, and in no way imply that nitrosylhemoglobin can be a donor of nitric oxide or its biological equivalent, as the affinity of the heme Fe for NO is extremely high. There can be no motivation drawn from the Moore or Sharma references to use nitrosylhemoglobin, either alone or in combination, for delivering NO or for scavenging oxygen free radicals, as nitrosylhemoglobin was not known to deliver NO or to have any physiological effects as a vasodilator, an oxygen carrier, or as a scavenger of NO· or oxygen free radicals. The prior art taught that nitrosylhemoglobin is oxidized in the presence of oxygen to form methemoglobin and nitrate. See reference AX.

Combining the cited references, one of ordinary skill in the art would conclude that Moore and Sharma do not teach anything relevant about the delivery of NO in a mammal, or about scavenging NO· or oxygen free radicals, as nitrosylhemoglobin cannot carry out these functions. Assessing the teachings of Stamler (WO 93/09806), one of ordinary skill in the art might think that a low molecular weight S-nitrosothiol or a SNO-protein such as SNO-albumin should be made and tested for the purpose of delivering NO. Either of these might be added to the cross-linked hemoglobin blood substitute described by Feola *et al.* However, at the same time, one of ordinary skill might also have wondered what physiological effect small (nanomolar) amounts of an S-nitrosothiol donor of NO might have, if any, in the presence of a large excess of hemoglobin (millimolar amounts) which was known to *bind* NO on the heme Fe, promoting platelet deposition and blocking vasodilation otherwise induced by NO. See, for example, Marcus, A.J. *et al., Circulation 93*:208-209, 1996, third paragraph; copy provided as Exhibit V. One of ordinary skill in the art, knowing that the effect of introducing donors of NO intravenously is to cause methemoglobinemia, might, in fact, be deterred from trying methods of therapy with new agents to be used in the delivery of NO.

Rejection of Claims 24-26 Under 35 U.S.C. § 103(a)

Claims 24-26 have been rejected under 35 U.S.C. § 103(a) "as being unpatentable over Feola et al. and Stamler, and if necessary further in view of Moore or Sharma, as applied to Claims 17-19 and 26 above, and further in view of Chem. Res. Tox. 1990 Vol. 3, pages 289-291."

Applicants respectfully request clarification of the reasoning behind the combination of references cited in the rejection, as it is not understood what "if necessary" means. If there are alternatives in the reasoning, Applicants request that they be written out, so that there is opportunity for Applicants to respond sufficiently to each rejection.

The teachings of Feola *et al.* (US 5,439,882), Stamler (WO 93/09806), Moore *et al.*, (*J. Biol. Chem. 251(9)*:2788-2794, 1976), Sharma *et al.*, (*J. Biol Chem. 253*:6467-6472, 1978) and Wade, R.S. and C.E. Castro (*Chem. Res. Tox. 3*:289-291, 1990) have been described above.

The Examiner states, "As discussed above, and as hereby incorporated by reference in its entirety, the Stamler reference suggests the use of nitrosylated hemoglobin alone or combined with a thiol containing compound in order to function equivalently to the Feola hemoglobin preparation as a blood substitute useful to treat sickle cell anemia."

The Examiner seems to assume that Stamler (WO 93/09806) teaches nitrosylated hemoglobin. The reference discusses SNO-hemoglobin as if it had been synthesized, but, as presented above and in the previous Amendment, and as presented in the Declaration of Jonathan S. Stamler, M.D., Under 37 C.F.R. § 1.132, previously mailed to the Patent Office on January 6, 1999, SNO-hemoglobin was not produced at that time. No physiological effects of SNO-hemoglobin were demonstrated, could have been demonstrated at that time, or could have been correctly predicted. Applicants can find no teaching or suggestion, either in WO 93/09806 or in Feola, that nitrosylated hemoglobin be combined with a thiol, as in Claim 25, for any purpose.

The point of the extensive discussion by the Examiner regarding different methods and a variety of conditions in WO 93/09806 to ostensibly produce S-nitrosohemoglobin is not understood. Methods of reportedly producing S-nitrosylhemoglobin, beyond attempting to show the existence of S-nitrosylhemoglobin, do not contribute to the obviousness of a method for treating a human with sickle cell anemia comprising administering to the human an effective amount of a preparation comprising SNO-Hb(FeII)O_2.

The Examiner additionally states, ". . . S-nitrosylation of hemoglobin serves to increase hemoglobin-oxygen binding as taught by Stamler *et al.* (e.g., see pages 19-20)."

The Stamler reference WO 93/09806 does not report any credible data to show that S-nitrosylated hemoglobin was, in fact, made. The Stamler reference also does not, and cannot, report any effects of S-nitrosylation on hemoglobin. If, in fact, S-nitrosylated hemoglobin were to increase hemoglobin-oxygen binding, one of ordinary skill in the art would be discouraged from pursuits to make and use this product, as a hemoglobin that cannot release oxygen to tissues in need of it would be detrimental to health.

The Examiner cites a further reference, stating, "Further, the Chem. Res. Tox. 1990 Vol. 3, pages 289-291 discloses a method of transferring the nitrosyl group to sulfur (as well as oxygen, nitrogen and sulfur) of heme proteins, including hemoglobin to thus form SNO-hemoglobin, and thus form thionitrosylated hemoglobin compositions."

As also stated in the above section of this Amendment, Wade, R.S. and C.E. Castro (*Chem. Res. Tox. 3*:289-291, 1990) describe a reaction of nitrosoamines with heme to produce nitrosyl-heme products (Equation 1), a reaction of hemoglobin or myoglobin with nitric oxide to produce FeII-NO adducts (first paragraph), a reaction of metmyoglobin with nitric oxide to produce the heme-NO adduct (Equation 2), and the corresponding heme-NO adducts of the other heme proteins listed in Table 1 on page 289. Wade and Castro also describe (Equation 3) a reaction in which oxidized heme proteins, which are unable to carry oxygen, react with NO and a molecule providing a nucleophilic site (e.g., phenol), to produce a nucleophile-NO product (e.g., nitrosophenol), and the nitrosyl-heme protein. Nowhere does the reference describe a stable product of a reaction between a species of nitric oxide with a site on hemoglobin other than the heme Fe. The cited claims pertain to a method of therapy using SNO-hemoglobin, which is an S-nitrosyl product of the heme protein, not an Fe-nitrosyl product. Wade and Castro present no suggestion that SNO-hemoglobin is possible to produce, and no suggestion of how to produce any product other than a heme-NO product in which NO is bound to the heme Fe. Therefore, this reference can contribute nothing to the rejection, as it has nothing to do with S-nitrosohemoglobin, or the administration of S-nitrosohemoglobin, either with or without thiol or nitrosothiol in addition, or any method of treatment of sickle cell anemia.

Combining the references, one of ordinary skill in the art would assess the data in Example 19 of WO 93/09806 and conclude that SNO-hemoglobin was not made, but could

perhaps conclude that some SNO-protein such as SNO-albumin should be added to the cross-linked hemoglobin blood substitute of Feola *et al.*, for the expected advantage of vasodilation in a treatment of sickle cell anemia. One of ordinary skill in the art would conclude that the teachings of Moore, Sharma and of Wade and Castro are not related to a method of treating a human with sickle cell anemia, or to a method of delivering NO to tissues, or to SNO-Hb(FeII)O_2, and would not find a logical way to combine their teachings with those of WO 93/09806 and Feola *et al.*

CONCLUSION

The Examiner is respectfully requested to take into consideration the above amendments and remarks, and to withdraw the rejections. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,
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